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<b>(57) Abstract</b>  A polypeptide of up to 50 amino acids in length is described which is suitable for use as a vaccine against a streptococcal infection. The polypeptide comprises (a) the amino acids 150-168 of protein H of <i>S. pyogenes</i> having the sequence <u>QKQQQLETEKQISEASRKS</u> ; (b) an amino acid sequence for an outer membrane protein of a streptococcal strain corresponding to sequence (a); (c) a fragment of sequence (a) or (b) of six or more amino acids; or (d) a sequence comprising a sequence (a), (b) or (c) modified by deletion, insertion, substitution or rearrangement.			

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## PEPTIDES

### FIELD OF THE INVENTION

The invention relates to peptides and their use in vaccines against streptococcal infection.

### 5 BACKGROUND TO THE INVENTION

There are many *Streptococcus* species, responsible for causing disease states in humans and other animals.

Infections caused by streptococci can vary from uncomplicated suppurative diseases like pharyngitis and skin infections to severe diseases such as sepsis and toxic  
10 shock syndrome. Rheumatic fever and glomerulonephritis are serious nonsuppurative sequelae following acute *S. pyogenes* infections. Other diseases caused by streptococci include scarlet fever, impetigo, erysipelas, myositis, necrotizing fasciitis, septic arthritis, cellulitis, colonization and destruction of heart valves (endocarditis), neonatal  
15 infections, conjunctivitis, sinusitis, peritonitis, omphalitis, meningitis, abortion and chorioamnionitis, post-partum sepsis, upper respiratory tract disease in humans, pneumonia, otitis media, wound infections, abscesses, empyema, mastitis, urinary tract infections, osteomyelitis, strangles in horses, dental pathogens in humans, renal  
infections in humans.

Many bacterial infections such as acute pharyngitis are treatable with antibiotics.  
20 However, in certain areas of the world, antibiotic resistance, particularly to erythromycin, is becoming more common. Recently, there has been an increase in the incidence of acute rheumatic fever linked to streptococcal pharyngitis. Acute rheumatic fever has been associated with at least six different M-types of *S. pyogenes*. In addition, the number of cases of severe streptococcal infection has been rising, leading to  
25 bacteraemia and sepsis, necrotising fasciitis and myositis, puerperal sepsis and streptococcal toxic shock syndrome (STSS).

Prompt and aggressive treatment is essential in severe streptococcal infection. Presently, this treatment may encompass surgical debridement, antibiotic treatment, intravenous fluid, oxygen or ventilator support, dialysis, vasoconstrictives to elevate  
30 blood pressure, steroids and anti-thrombolytics. Even so, severe streptococcal infection can be fatal.

-2-

Present vaccine strategies for *S. pyogenes* have been focused on the outer membrane M-proteins. These proteins are considered to be key virulence factors in view of their ability to confer resistance to phagocytosis on the bacteria. Furthermore, these proteins provoke harmful host immune responses through their superantigenicity and their ability to induce cross-reactive antibody responses in humans. However, the variability and number of M-proteins and their ability to produce cross-reactive antibody responses have led to problems in the formulation of an effective vaccine against various serotypes of *S. pyogenes*.

There is a continuing need to develop additional treatment strategies for severe streptococcal infection and create new vaccine formulations which may be used to combat not only *Streptococcus pyogenes* infection but also infection caused by other streptococcus species.

#### SUMMARY OF THE INVENTION

The inventors have now established a link between clinical infection and virulence of *S. pyogenes* and the ability of *S. pyogenes* to aggregate. The ability to aggregate has been linked to an immunogenic region of protein H of *S. pyogenes*. Amino acid sequences corresponding to the amino acid sequence of this region can be found in the outer membrane proteins not only of a wide variety of *S. pyogenes* serotypes but also of many other streptococcal strains.

Accordingly, the present invention provides a polypeptide of up to 50 amino acids in length, suitable for use as a vaccine against streptococcal infection, comprising:

- (a) the amino acids 150-168 of protein H of *S. pyogenes* having the sequence QKQQQLETEKQISEASRKS;
- (b) an amino acid sequence of an outer membrane protein of a streptococcal strain corresponding to sequence (a);
- (c) a fragment of sequence (a) or sequence (b) of 6 or more amino acids; or
- (d) a sequence comprising the sequence (a), (b) or (c) modified by deletion, insertion, substitution or rearrangement.

The present invention also provides a chimeric protein comprising a first polypeptide having the sequence (a), (b), (c), (d) above and a second polypeptide which is not naturally contiguous to the first polypeptide.

-3-

In a further aspect, the present invention provides a polynucleotide encoding a polypeptide of the present invention.

The present invention also relates to expression vectors comprising a polynucleotide in the invention and regulatory sequences operably linked to said  
5 polynucleotide for expression of a polypeptide encoded by the polynucleotides and to host cells transfected with such expression vectors.

The present invention also provides a pharmaceutical composition comprising a polypeptide of the invention together with a pharmaceutical composition comprising a polypeptide of the invention together with a pharmaceutically acceptable carrier and a  
10 vaccine composition comprising a polypeptide of the invention, an adjuvant in a pharmaceutically acceptable carrier or a polynucleotide of the invention together with a pharmaceutically acceptable carrier for the polynucleotide.

The invention also relates to an antibody against a polypeptide according to the invention.

15 In a further aspect, the invention provides a polypeptide of up to 50 amino acids in length comprising:

(a) the amino acids 150-168 of protein H of *S.pyogenes* having the sequence QKQQQLETEKQISEASRKS;

(b) an amino acid sequence of an outer membrane protein of a streptococcal  
20 strain corresponding to sequence (a), said sequence having the ability to interfere with aggregation or adhesion of said streptococcal strain;

(c) a fragment of sequence (a) or (b) of six or more amino acids, which retains the ability to interfere with streptococcal aggregation or adhesion;

(d) a sequence comprising a sequence (a), (b) or (c) modified by deletion,  
25 insertion, substitution or rearrangement, the sequence retaining the ability to interfere with streptococcal aggregation or adherence.

## DESCRIPTION OF THE FIGURES

### Figure 1. Sedimentation analysis of AP1 bacteria

AP1 bacteria were grown at 37°C over night in TH (○), TH containing 10%  
30 human plasma (●) or TH containing 1.4 mg/ml human IgG (Δ). Bacteria were resuspended and left to settle at room temperature. The sedimentation rate was obtained

-4-

by plotting values of optical density at 620 nm against various time points.

**Figure 2.** Solubilization of AP1 surface proteins

(A) AP1 bacteria were incubated with PBS (○), papain (●), streptococcal cysteine proteinase (Δ) or CNBr (▲). Following digestion bacteria were centrifuged, washed, resuspended and analyzed for sedimentation.

(B) Bacterial cells as in (A) above, at a concentration of  $2 \times 10^9$  bacteria/ml, were serially diluted and tested for binding of  $^{125}\text{I}$ -labelled IgG.

(C) SDS-PAGE analysis of CNBr-solubilized material from AP1 bacteria. The fragments generated by CNBr are indicated in the schematic representations of protein H and M1 protein. The  $\text{NH}_2$ -terminal signal sequences (Ss) are indicated, and the proteins are associated with the bacterial cell wall through the COOH-terminal D domains. The sequences in the Ss, C and D domains show a high degree of homology. IgGFc-binding is located in the A-B domains of protein H and in the S domain of M1 protein. Numbers in the figure refer to amino acid residue positions. The protein H used herein is a truncated form (42-349) lacking the 27 COOH-terminal amino acid residues associated with the bacterial cell wall, whereas the M1 protein used covers residues 42-484

**Figure 3.** Protein H binds to AP1 bacteria and to purified protein H

(A) AP1 and AP6 bacteria,  $2 \times 10^9$  cells/ml, were serially diluted and tested for binding of  $^{125}\text{I}$ -labelled protein H (●) or M1 protein (Δ).  $^{125}\text{I}$ -labelled IgG (○) was used as a positive control.

(B) Various amounts of protein H and M1 protein were applied to PVDF filters. Filters were incubated with  $^{125}\text{I}$ -labelled protein H or M1 protein ( $2 \times 10^5$  cpm/ml) for 3 h and autoradiographed for 3 days.

**Figure 4.** Mapping of the self-associating region in protein H

(A) Binding of  $^{125}\text{I}$ -labelled protein H (○), fragment AB (Δ) or fragment A of protein H (□) to AP1 bacteria.

(B) The binding of  $^{125}\text{I}$ -labelled protein H to AP1 bacteria ( $2 \times 10^9$  cells/ml) was inhibited with various amounts of unlabelled protein H (○) or with fragments AB (Δ) and A (□) of protein H.

(C) Schematic figure of protein H. The various protein H fragments are

-5-

indicated below the figure together with the sequence for the aggregative protein H peptide (APP). Numbers refer to amino acid residues.

**Figure 5.** Radiolabelled protein H has affinity for protein H-Sepharose.

$^{125}\text{I}$ -labelled protein H ( $10^6$  cpm) was applied to a protein H-Sepharose column.

5 The column was extensively washed with PBSAT, eluted with human IgG (5 mg/ml) and, after a second wash with PBSAT, finally eluted with 3 M KSCN. The radioactivity of the fractions were plotted against the volume.

**Figure 6.** APP is cross-linked to protein H.

Protein H (300 pmol) was crosslinked with DSS in the presence of  $^{125}\text{I}$ -labelled  
10 APP (approximately 450 pmol). Samples were analyzed by SDS-PAGE, one gel was stained and one gel was dried and autoradiographed. Lane 1: protein H without crosslinker; Lane 2: protein H with crosslinker; Lane 3: protein H crosslinked in the presence of  $^{125}\text{I}$ -APP; Lane 4: protein H crosslinked in the presence of  $^{125}\text{I}$ -APP and excess amount of unlabelled APP (450 nmol); Lane 5: protein H crosslinked in the  
15 presence of  $^{125}\text{I}$ -labelled B1 domain of protein L.

**Figure 7.** APP-related sequences are found in several M and M-like proteins

Alignment of sequences related to APP found in the data base. Identity to APP at the amino acid and the nucleotide level is given together with the M serotype from  
20 which the protein originates. Indicated is also the position of the APP-related sequence in the various proteins M49, Sir22, ML2.1, M1, M5, M12, Arp4 and M6.

**Figure 8.** Analysis of *S. pyogenes* sedimentation and adherence

(A) Wild-type AP1, mutant lacking protein H (BM27.6), mutant lacking both protein H and M1 protein (BMJ71), wild-type M6 strain (JRS4) (which does not  
25 express protein H), and mutant lacking M6 protein (JRS145), were tested for aggregation. The sedimentation was measured as decrease in optical density at 620 nm after 1 h. Mean values  $\pm$  SD are given.

(B) Wild-type strains and mutants were tested for their adherence to human epithelial cells. One hundred per cent adherence correspond to  $1.49 \times 10^6 \pm 0.5$  AP1  
30 bacteria/ epithelial cell layer or  $0.27 \times 10^6 \pm 0.04 \times 10^6$  JRS4 bacteria/epithelial cell layer. The AP1 mutants are compared to AP1, the JRS145 mutant is compared to JRS4.

-6-

Values are mean  $\pm$  SD.

**Figure 9.** Results of ELISA using antiserum prepared by injecting APP conjugated to KLH together with adjuvant into rabbits.

**Figure 10.** Nucleotide sequence of APP-peptide (33-mer) and homology to other M proteins.

**Figure 11.** Alignment of APP-related sequences using APP-peptide (33-mer)

**Figure 12.** Effect of pre-adsorption with peptides on detection of antibodies induced by Spy-PH-YQE33 peptide. Sheep were primed and boosted with Protein H-derived peptide Spy-PH-YQE33 conjugated to KLH carrier protein. Post-immune sera (1:10000 dilution) were incubated (60 min, 37°C) with 100 µg/ml irrelevant peptide (Spn-LP-KEY17) or 100 µg/ml relevant peptide (Spy-PH-QKQ19). Control samples were incubated in the absence of peptide. Antibodies against Spy-PH-YQE33 epitopes were then determined by ELISA. Results are expressed as optical density (OD) measured at 450 nm (mean, n=4 wells).

#### DETAILED DESCRIPTION OF THE INVENTION

A polypeptide of the invention is one of up to 50 amino acids in length, suitable for use as a vaccine against a streptococcal infection. The polypeptide may consist essentially of (a) the amino acids 150-168 of Protein H of *S.pyogenes* having the sequence QKQQQLETEKQISEASRKS. A polypeptide of the invention may also comprise (b) an amino acid sequence of an outer membrane protein of a streptococcal strain corresponding to sequence (a), a fragment of either sequence (a) or (b) of six or more amino acids in length; or (d) a sequence comprising a sequence (a), (b) or (c) modified by deletion, insertion, substitution or rearrangement.

Streptococci have been classified into various groups under the Lancefield classification. This classification is based on group-specific antigens in the cell wall polysaccharide. Key Lancefield groups are Group A comprising *Streptococcus pyogenes*, Group B comprising *Streptococcus agalactiae*, Group C and G including *Streptococcus equi* and *Streptococcus equisimilis*, Group D including *Enterococcus faecalis* and Group Viridian including *Streptococcus mutans* and *Streptococcus sanguis*. A non-groupable species is *Streptococcus pneumoniae*.



-7-

Within each group, bacteria may be further classified according to their serotype. For Group A Streptococcus, serotyping is carried out by reference to additional surface antigens, the M proteins. These proteins divide the group A Streptococcus into more than 80 serotypes. Some M serotypes are associated with increased virulence and pathogenicity. In particular, M1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 17, 18, 19, 22, 24, 49, 55, 57 and 60 serotypes are associated with more serious infection in humans.

*S.pyogenes* can express a number of other outer membrane proteins including immunoglobulin binding proteins. Protein H is an example of an outer membrane protein expressed by M1 serotypes of *S. pyogenes*. Protein H, along with other IgG binding proteins expressed by *S. pyogenes*, is structurally related to M proteins which may also bind IgG, and now considered to be part of the same family. Nielson *et al* Biochemistry 1995 Vol. 34 No. 41 13688-13698 describes the structure of protein H and M1 protein of AP1 strain of *S. pyogenes* and sets out the full sequence and domain structure of these proteins.

Some streptococcal strains have been shown to aggregate, with a greater ability to aggregate being associated with an increase in the virulence of the bacteria. The examples set out below demonstrate the role of protein H in aggregation, through protein H - protein H interactions. This aggregative activity has been mapped to the sequence (a) above comprising residues 150-168 of protein H and referred to herein as APP. APP is suitable for use as a vaccine against streptococci in view of its role in the aggregative properties associated with virulence of the bacteria. Similarly the sequence of residues 145-177 of protein H encompassing the sequence (a), namely sequence YQEQQLKQKQQLETEKQISEASRKSLSRDLEASR is of particular interest, and is referred to herein as a 33-mer.

Sequence comparison studies with outer membrane proteins of a broad range of *S. pyogenes* serotypes show that there are related sequences to be found in many other *S. pyogenes* strains. Furthermore, similar sequences were observed in non-group A streptococci. Accordingly the invention relates to the sequence (b) comprising a corresponding sequence of an outer membrane protein of a streptococcal strain. This includes those proteins expressed on the surface of Groups A, B, C, D, G and Viridian

-8-

streptococcus. In a preferred embodiment of the invention, the sequence (b) is derived from an outer membrane protein of Group A Streptococcus, *S.pyogenes*. In a preferred embodiment, the amino acid sequence is derived from one of the more virulent serotypes of *S.pyogenes* namely M1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 17, 18, 19, 22, 24, 49, 55, 57 and 60. The outer membrane protein may for example comprise one of the M proteins of Group A Streptococcus. Examples of other proteins include protein Arp and protein Sir22 expressed by some streptococcal strains which are associated with binding of plasma proteins. The relevant outer membrane proteins and sequences can be elucidated by using APP or the 33-mer and establishing the best possible alignment when looking at the sequences of outer membrane proteins of streptococci.

Examples of these sequences are given in Figures 7 and 11. Examples of these sequences are set out below:

QKQQQLEKEKQISEASRKS, IEKAKLEEEKQISDASRQS,  
 AEQQKLEEQNKISEA SRKG, GQIKQLEEQNKISEASRKG,  
 AEHQKLKEEKQISDASRQG, AELDKVKEEKQISDASRQG,  
 YQEQLQKQQQLEKEKQISEASRKSLSRDLEASR,  
 YQEQLQKQQQLEKEKQISEASRKSLSRRDLEASR,  
 YKEQLHKQQQLETEKQISEASRKSLSRDLEASR,  
 KKELEAEHQKLKEEKQISDASRQGLSRDLEASR,  
 KEQLTIEKAKLEEEKQISDASRQSLRRDLASR,  
 KKQLEAEQQKLEEQNKISEASRKGSLRRDLASR,  
 LAEKDGQIKQLEEQNKISEASRKGTDLEAVR,  
 KKQLEAEHQKLEEQNKISEASRQSLRRDLASR, or  
 LANLTAELDKVKEEKQISDASRQGLRRDLASR.

As can be seen from the examples given in Figure 7, some of the more virulent strains of streptococci identified above have sequences which correspond to sequence (a) which emphasises the suitability of the sequences as vaccines. Other corresponding sequences may include sequences within the C2 and C3 repeat domains of protein H having a high degree of homology with APP.

Preferably, a polypeptide of sequence (b) or (d) of the invention will be at least 40% homologous to the sequence (a) or the 33-mer identified above over its entire

-9-

length, based on amino acid identity. More preferably, the sequence (b) or (d) of the invention is at least 50% and more preferably at least 60%, 70% or 80% homologous to the sequence (a). More preferably, the sequence (b) or (d) comprises the sequence that is at least 90% and more preferably at least 95%, 97% or 98% homologous to the sequence (a) or the 33-mer identified above.

As an alternative approach, the presence of a corresponding sequence may be established through hybridisation using the nucleotide sequences set out in Figure 10, as discussed in more detail below.

The invention also relates to fragments of the sequences (a) and (b) of six or more amino acids in length, preferably 8 or more amino acids or 10 or more amino acids in length. This fragments may be up to 10 amino acids in length preferably up to 12, 16 or 18 amino acids in length.

Amino acid substitutions, modifications deletions or rearrangements may be made to the sequences (a), (b) or (c) for example at from 1, 2, 3, 4, 5 up to 9 amino acids of sequence (a) preferably, at from 1, 2 or 3 amino acids. Conservative substitutions may be made, for example according to the following Table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Other preferred substitutions can be established for example by comparison of the conserved sequences as defined above. Additionally, other substitutions seen in more than one of the conserved sequences set out above may be considered to be

-10-

preferred.

Except where specified to the contrary, the polypeptide sequences described herein are shown in the conventional one-letter code and in the N-terminal to C-terminal orientation. The amino acid polypeptides of the invention may also be  
5 modified to include non naturally-occurring amino acids or to increase the stability of the compound *in vivo*. When the compounds are produced by synthetic means, such amino acids may be introduced during production. The compound may also be modified following either synthetic or recombinant production.

Polypeptides of the invention may also be made synthetically using D-amino  
10 acids. In such cases, the amino acids will be linked in a reverse sequence in the C to N orientation. This is conventional in the art for producing such peptides.

A number of side-chain modifications for amino acids are known in the art and may be made with the side-chains of polypeptides of the present invention. Such  
15 modifications include for example, modifications of the amino acid group by reductive alkylation by reaction with an aldehyde followed by reduction with  $\text{NaBH}_4$ , amidination with methyl acetimidate, for acylation with acetic anhydride. The carboxy terminus and any other carboxy side-chains may be blocked in the form of an ester group, e.g. a  $\text{C}_{1-6}$  alkyl ester.

The above examples and modifications to amino acids are not exhaustive. Those  
20 of skill in the art may modify amino acid side-chains where desired using chemistry known *per se* in the art.

The polypeptide of the invention may consist essentially of the sequences (a), (b), (c) or (d).

In general, the polypeptides of the present invention are selected or modified to  
25 maintain their suitability as vaccine compositions. As will be well appreciated by those skilled in the art, a polypeptide suitable for use as a vaccine composition in accordance with the invention is a polypeptide which is able to generate a protective immune response against a streptococcal infection. The polypeptides of the present invention can typically be selected by the ability to interfere with bacterial aggregation or  
30 adhesion to epithelial surfaces. In the context of aggregation and adhesion, the sequence (a) may be used as an antiaggregative agent. The corresponding sequence (b)

-11-

may be used to inhibit aggregation of the streptococcal strain from which the sequence derives. In the alternative, the sequences may interfere generally with streptococcal aggregation and/or adhesion of the bacteria to epithelial cells. In this context, aggregation of the bacteria means that the aggregation seen in streptococci as exemplified in Example 1 and measured in absorbance studies. Preferably, the peptide will reduce aggregation by 30% more preferably by at least 50%. In addition or in the alternative, the polypeptide may interfere with adhesion of the bacteria to epithelial cells as exemplified in Example 4.

Fragments of sequences (a) and (b) may also be selected on the basis of their ability to interfere with bacterial aggregation or adhesion. Modifications, substitutions, deletions or rearrangements as outlined above may also be selected to maintain the ability of the polypeptide to interfere with bacterial aggregation.

Thus, in an alternative aspect of the invention, we provide a polypeptide of up to 50 amino acids in length comprising:

(a) the amino acids 150-168 of protein H of *S. pyogenes* having the sequence QKQQQLETEKQISEASRKS;

(b) a corresponding sequence to sequence (a) of an outer membrane protein of a streptococcus strain, said sequence having the ability to interfere with aggregation or adhesion of said streptococcal strain;

(c) a fragment of sequence (a) or (b) of 6 or more amino acids, which retains the ability to interfere with streptococcal aggregation or adhesion; and

(d) a sequence comprising sequences (a), (b) or (c) modified by deletion, insertion, substitution or rearrangement, the sequence retaining the ability to interfere with streptococcal aggregation or adherence.

The polypeptides of the present application are thus also proposed for use to inhibit bacterial aggregation or adherence to epithelial cells and thus may be used to reduce virulence of the bacteria.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially

-12-

purified form, in which case the polypeptide of the invention will generally comprise 90%, e.g. more than 95% or more than 99% by weight of the polypeptide in the preparation produced by the isolation or purification procedure.

Polypeptides of the invention may be made synthetically or recombinantly using techniques which are widely available in the art. Synthetic production generally involves stepwise addition of individual amino acid residues to a reaction vessel in which a polypeptide of a desired sequence is being made. Examples of recombinant techniques are described below.

The polypeptides of the present invention are up to 50 amino acids in length. More preferably, the polypeptide is of no more than 40 to 45 amino acids in length, and most preferably up to 30, 20 or 15 amino acids in length.

The invention also relates to chimeric proteins comprising a first polypeptide in accordance with the invention and a second polypeptide which is not naturally contiguous to said first polypeptide. Thus, the polypeptide of the invention may comprise repeats of the sequences (a), (b), (c) or (d) or combinations thereof. The polypeptide may be cyclized. Alternatively the polypeptide may comprise separate repeat regions linked through a linker sequence such as a polylysine bridge. The peptides may also be formulated as fusion proteins with a carrier, particularly when used as a vaccine with carriers such as keyhole limpet hemacyanin, diphtheria toxoid, tetanus toxoid.

The polypeptides of the invention may be formulated into pharmaceutical compositions. The compositions comprise the polypeptides together with a pharmaceutically acceptable carrier or diluent. Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for oral, topical or parenteral (e.g. intramuscular or intravenous) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy.

For example, formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may

-13-

include suspending agents and thickening agents, and liposomes or other micro-particulate systems which are designed to target the polypeptide within the body.

The peptides of the invention may also be used to generate an immune response to provide protection against later infections by Streptococcus.

5 Means of presentation of the peptide immunogen(s) include, but are not restricted to: free peptides, as peptides conjugated to suitable carrier proteins such as keyhole limpet haemocyanin, bovine serum albumin, ovalbumin, inactivated bacterial toxins such as tetanus and diphtheria toxoids, bacterial or mammalian heat shock proteins; as fusion or chimeric proteins, expressed by recombinant bacteria or viruses  
10 comprising the peptide of choice and one or more peptides or proteins containing T- or B-cell epitopes, or specific binding domains for molecules present on the lymphocyte surface; as peptides or fusion proteins expressed on the surface of, or secreted by, live bacterial or viral vectors.

Peptides may also be entrapped within, or presented on the surface of,  
15 liposomes; be incorporated into biodegradable microspheres formulated from poly (D,L) lactic co-glycolic acid or other polymers, or presented on the surface of micelles formulated from saponins such as Quil A, cholesterol and phospholipid suitable detergent.

Multiple copies of the peptide may be administered to enhance the immune  
20 response as synthetic polypeptides containing between 2 and 20 copies of the peptide vaccinogen and/or single or multiple copies of additional T- or B-cell epitopes. Alternatively, between 2 and 20 copies of the peptide vaccinogen and/or multiple copies of additional T- and B-cell epitopes may be presented as chemically synthesised branched oligomers formed around a core matrix of lysine or another amino acid.

25 Peptides used for vaccination may be chemically modified to facilitate conjugation to protein carriers and/or to increase their immunogenicity. Suitable modifications include, but are not confined to, the addition of cysteine residues at each terminus to permit polymerisation via disulphide bond formation. Peptides may be modified to increase their immunogenicity by conjugation to lipids such as  $\alpha$ -  
30 aminohexadecanoic acid at the N-terminus.

The preparation of vaccines which contain an immunogenic polypeptide(s) as

-14-

active ingredient(s), is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The  
5 active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like in combinations thereof.

In addition, if desired, the vaccine may contain minor amounts of auxiliary  
10 substances such as wetting or emulsifying agents, pH buffering agents and/or adjuvants which enhance the effectiveness of the vaccines. Examples of adjuvants which may be effective include but are not limited to: alum or other aluminum salts, calcium salts; water-in-oil emulsions containing mineral oil, squalene or squalane; oil-in-water emulsions of squalene, squalene or oils in combination with surfactants such as Tween  
15 80 or Span 85. These emulsions may also include components such as N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP), or derivatives of synthetic sulpholipopolysaccharides, or non-ionic block copolymers; compositions containing saponins such as Quil A and/or monophosphoryl lipid A (MPL); carbohydrate polymers such as mannan or beta 1-3 glucose; natural or recombinant bacterial toxins such as  
20 cholera toxin or Escherichia coli labile toxin; natural or recombinant cytokines such as human interleukin-1 (IL-1), IL-2, IL-4 or IL-12. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic peptide resulting from administration of this peptide in vaccines which are also comprised of the various adjuvants.

25 The vaccines are conventionally administered parenterally by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations or topical application to the nasal, rectal or vaginal mucosa, or through inhalation of a liquid or powder formulation. For suppositories, traditional binders and  
30 carriers may include for example, polyalkylene glycol triglycerides. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of



-15-

mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension.

Capsules, tablets and pills for oral administration to a patient may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate, cellulose acetate phthalate or hydroxypropylmethyl cellulose.

The polypeptides of the invention may be formulated into the vaccines as natural or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino acid groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric and maleic. Salt forms for the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxide and such organic bases as isopropylamine and trimethylamine, 2-ethylamino ethanol, histidine and procaine.

Peptide vaccinogens may be given as single or repeat doses, with each dose consisting of between 2 and 5000 micrograms, preferably between 10 and 1000 micrograms of the peptide. Preferably two or more repeat doses will be given.

When formulating vaccine compositions, additional antigenic components derived from streptococcal proteins may be incorporated. For example, streptococcal extracellular cysteine proteinase (SCP) may be incorporated in the vaccine formulation or an antigenic fragment thereof. SCP is thought to play an important role in the virulence of *S. pyogenes*. *S. pyogenes* which are deficient in active SCP production have been shown to lose virtually all their virulence. Synthetic peptides such as those described in WO96/08569 representing conserved immuno-dominant epitopes within SCP may readily be incorporated in a suitable vaccine composition.

Another streptococcal protein which may be useful in a vaccine formulation is the streptococcal inhibitor of complement-mediated lysis, namely protein SIC. Again, this protein plays a role in *S. pyogenes* pathogenicity and virulence and is described in WO97/13786.

-16-

As outlined above, the peptides of the invention may be made by recombinant techniques. Thus, the invention also provides nucleic acids encoding polypeptides of the invention. Particularly preferred are the polynucleotides set out in Figure 10 for the 33-mer sequence or fragments thereof encoding the 19-mer APP sequence (a).

5 A polynucleotide of the invention is capable of hybridising selectively with the coding sequence of sequence (a) for example the sequence set out in Figure 10 for the 33-mer sequence or the fragment thereof encoding the 19-mer APP sequence (a), or to a sequence complementary to that coding sequence. Polynucleotides of the invention include variants of the sequence shown in Figure 10 which encode the amino acid  
10 sequences of APP or the 33-mer and variants thereof which encode the polypeptide having the sequence (b), (c) or (d) of the invention. Typically, a polynucleotide of the invention is a contiguous sequence of nucleotides which is capable of selectively hybridising to the polynucleotide sequence of the 33-mer or 19-mer as set out in Figure 10 or to the complement of that sequence.

15 A polynucleotide of the invention hybridising to the sequence of Figure 10 encoding the 19-mer APP sequence (a) can hybridise at a level significantly above background. Background hybridisation may occur, for example, because of other DNAs present in a DNA library. The signal level generated by the interaction between a polynucleotide of the invention and the polynucleotide sequence of Figure 10  
20 encoding the 19-mer sequence (a) is typically at least 10 fold, preferably at least 100 fold, as intense as interactions between other polynucleotides and the polynucleotide sequence of Figure 10 encoding sequence (a). The intensity of interaction may be measured, for example by radiolabelling the probe e.g. with  $^{32}\text{P}$ . Selective hybridisation is typically achieved using conditions of medium to high stringency, for example, 0.03  
25 M sodium chloride and 0.03 M sodium citrate at from about 50°C to about 60°C.

A nucleotide sequence capable of selectively hybridising to the DNA coding sequence of sequence (a) or to the sequence complementary to that coding sequence will be generally at least 50%, preferably at least 70 or 80% and more preferably at least 90 or 95% homologous to the polynucleotide of Figure 10 or its complement over a  
30 region of at least 30, preferably at least 40, for instance, at least 60, 80 or 90 contiguous nucleotides over the entire length of the polynucleotide sequence set out in Figure 10

-17-

for the 33-mer sequence. Methods of measuring polynucleotide homology are well known in the art. The UWGCG package, which provides the BESTFIT program can be used to calculate homology, e.g. on its default settings (Deveraux *et al*, Nucl. Acids. Res. 12, 387-395, 1984).

5 Any combination of the above mentioned degrees of homology and minimum size may be used to define polynucleotides of the invention, with the more stringent combinations, i.e. higher homology over longer lengths being preferred.

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible  
10 host cell. Thus, in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*,  
15 yeast, mammalian cell lines and other eukaryotic cell lines, for example, insect SF9 cells.

Preferably, the polynucleotide of the invention in a vector is operably linked to a regulatory sequence which is capable of providing for the expression of the coding sequence of the host cell, i.e. the vector is an expression vector. The term operably  
20 linked refers to a juxtaposition where the components described are in a relationship permitting them to function in their intended manner. A regulatory system operably linked to a coding sequence is located in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

Such vectors may be transformed or transfected into a suitable host cell as  
25 described above to provide for expression of a polypeptide of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide expression by the vector of a coding sequence encoding the polypeptide, and optionally recovering the expressed polypeptide.

30 The vector may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and

-18-

optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, yeast promoters include *S. cerevisiae* GAL4 and ADH promoters. Viral promoters include the SV40 large T antigen promoter, retroviral LTR promoters and adenovirus promoters. All these promoters are readily available in the art.

The nucleotide sequences of the invention and expression vectors can also be used as vaccine formulations as outlined above. The vaccines may comprise naked nucleotide sequences or in combination with cationic lipids, polymers or targeting systems.

The immunogenic polypeptides prepared as described above can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide of the invention. Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to the polypeptide contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art.

Monoclonal antibodies directed against Streptococcal epitopes in the polypeptides of the invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against polypeptides of the invention can be screened for various properties; i.e., for isotype and epitope affinity.

Antibodies, both monoclonal and polyclonal, which are directed against

-19-

polypeptides of the invention are particularly useful in diagnosis, and those which are neutralising are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotypic antibodies. Anti-idiotypic antibodies are immunoglobulins which carry an "internal image" of the antigen of the infectious agent against which protection is desired.

Techniques for raising anti-idiotypic antibodies are known in the art. These anti-idiotypic antibodies may also be useful for treatment of Streptococci, as well as for an elucidation of the immunogenic regions of polypeptides of the invention.

It is also possible to use fragments of the antibodies described above, for example, Fab fragments.

#### EXAMPLES

##### **Example 1** - *Surface proteins promote aggregation of AP1 bacteria*

*S. pyogenes* bacteria of the AP1 strain (40/58) from the WHO Collaborating Centre for Reference and Research on Streptococci, Prague, Czech Republic, were found to aggregate following growth overnight at 37°C in Todd Hewitt broth (TH) (Difco, Detroit, MI). These visible aggregates rapidly fall to the bottom of the testtube. By measuring optical density at 620 nm at various time intervals, the degree of aggregation was determined. Human plasma (10% solution) or human IgG (1.4 mg/ml) (Sigma Chemical Co., St. Louis, MO) present during growth led to no aggregation being seen and much slower settling of cultures (Fig. 1). Microscopic analysis of the cultures revealed large aggregates of bacteria except in those containing plasma or IgG, where short chains and almost no aggregates could be seen (not shown).

At the bacterial surface protein H and M1 protein are responsible for IgG-binding, and a possible role for these molecules in the formation of aggregates was proposed.

Papain and a cysteine proteinase produced by *S. pyogenes* can remove proteins H and M1 from the surface of AP1 bacteria. AP1 bacteria were suspended in 0.01 M Tris-HCl, pH 8.0, to 1% (v/v) ( $2 \times 10^9$  cells/ml). The bacteria were incubated with papain (Sigma) and L-cystein (100 µg papain and 28 µl 1M L-cystein/ml cell solution) at 37°C for 1 h. Iodoacetic acid (Sigma) was added to a final concentration of 10 mM to terminate the digestion. Bacteria were collected by centrifugation at 3000 x g, washed

-20-

twice with PBS and submitted to sedimentation analysis. Binding of  $^{125}\text{I}$ -labelled IgG to the cells was also performed. For digestion with streptococcal cysteine proteinase, 0.5 ml AP1 bacteria ( $2 \times 10^{10}$  cells/ml PBS) were incubated with 5  $\mu\text{g}$  of the activated enzyme for 3 h at  $37^\circ\text{C}$ . The enzyme was inactivated by the addition of iodoacetic acid to 6 mM and cells were washed twice with PBS and analyzed for sedimentation and binding of  $^{125}\text{I}$ -labelled IgG.

Following treatment with these enzymes or cyanogen bromide (CNBr), the bacterial suspensions settled slowly and no longer showed IgG-binding activity (Fig. 2A, B). Peptides solubilized with CNBr were separated by SDS-PAGE and gave rise to bands with apparent molecular masses of 54, 49 and 44 kDa, respectively. These bands, denoted I, II and III in Figure 2C, were subjected to  $\text{NH}_2$ -terminal amino acid sequencing. The sequences of bands I and II were determined to Asn-Gly-Asp-Gly-Asn and Glu-Val-Ala-Gly-Arg, sequences that start at positions 42 and 82, respectively, in M1 protein, whereas the  $\text{NH}_2$ -terminal sequence of band III (Glu-Gly-Ala-Lys-Ile) corresponds to a protein H fragment starting at position 42. The size of the fragments generated with CNBr correspond well with the positions of methionine residues in the M1 protein and protein H sequences. These data, together with the observations concerning IgG-binding and aggregation, indicate that protein H and/or M1 participate in cell-cell interactions resulting in the formation of large bacterial aggregates.

#### **Example 2** *Protein H binds to itself*

Radiolabelled protein H or M1 protein, labelled with  $^{125}\text{I}$  using the Bolton and Hunter reagent (Amersham, UK), was incubated with AP1 bacteria in TH as before. Protein H was found to bind to the bacterial cells (Fig. 3A). The AP6 strain of the M6 serotype is a non-protein H-expressing strain and neither protein H nor M1 protein showed affinity for these bacteria. Included in Figure 3A, as a positive control, are also the binding curves obtained with  $^{125}\text{I}$ -labelled IgG. The binding of protein H to AP1 bacteria could be inhibited with protein H efficiently (see below, Fig. 4B). Protein H and M1 protein were also applied in slots to PVDF membranes and probed with  $^{125}\text{I}$ -labelled protein H or M1 protein. An interaction could be seen between the protein H molecules, whereas M1 protein bound neither protein H nor M1 protein itself (Fig. 3B). Furthermore, protein H immobilized on Sepharose was also found to specifically

-21-

interact with  $^{125}\text{I}$ -labelled protein H (see below). These observations suggest that protein H, through homophilic interactions, contributes to AP1 aggregation.

**Example 3** *Identification of a self-interacting region in protein H*

By using various protein H fragments, the binding sites on the protein H molecule for IgG, albumin, and FNIII domains have been determined. For IgG and FNIII the binding sites are located within the  $\text{NH}_2$ -terminal AB and A region respectively, whereas the binding of albumin resides in the C repeats of protein H. Figure 4C shows a schematic representation of protein H, where additionally the fragments used in the experiments below are indicated.

Radiolabelled protein H and fragment AB, but not fragment A, showed affinity for AP1 bacteria (Fig. 4A). The binding of radiolabelled protein H to AP1 bacteria could also be blocked with fragment AB but not with fragment A, suggesting that the binding is located in the B region (Fig. 4B). Nevertheless, compared to protein H a larger amount of AB was required for inhibition. Thus, to obtain 50% inhibition, about 300-fold more of the AB fragment was needed. Moreover, fibronectin and albumin could not block the binding of protein H to AP1 bacteria (data not shown), which also suggests that B represents the self-associating region.

IgG-binding to protein H has been mapped to a region covering the  $\text{NH}_2$ -terminal part of B and the  $\text{COOH}$ -terminal part of A. The fact that IgG interferes with bacterial aggregation indicated that the self-associating region could overlap the IgG-binding site. However, when radiolabelled protein H was applied to a column of protein H-Sepharose, the bound radioactivity could not be eluted with an excess of unlabelled IgG (Fig. 5). In contrast, the radioactivity was readily eluted with 3 M KSCN. The protein H fragments were also tested for binding to protein H-Sepharose, and again only the AB fragment had affinity (not shown). This fragment includes also the 10  $\text{NH}_2$ -terminal amino acid residues from the C1 repeat (see Fig. 4C), which raised the possibility that this sequence and/or the  $\text{COOH}$ -terminal part of B represents the self-associating region. A peptide covering this region was therefore synthesized (Fig. 4C) and designated APP; aggregative protein H peptide 150-168.

At the bacterial surface protein H and other members of the M protein family form  $\alpha$ -helical coiled-coil dimers. The homobifunctional cross-linker disuccinimidyl

-22-

suberate (DSS) from Pierce in Me<sub>2</sub>SO<sub>4</sub>, was added to a final concentration of 1mM to protein solutions in PBS for 30 min. at 4°C. 1M Tris-HCl pH7.5 and 1M NaCl were added to terminate the reaction. DSS was found to dimerize protein H in solution (Fig. 6, stain, lanes 1 and 2). To test if APP could interact with the dimers, protein H was incubated with <sup>125</sup>I-labelled APP in the absence or presence of a 1000-fold molar excess of unlabelled APP, followed by cross-linking with DSS and SDS-PAGE. One gel was stained (Fig. 6, stain) and one was dried and autoradiographed (Fig. 6, autoradiogram). The <sup>125</sup>I-labelled APP preferentially bound to protein H dimers (Fig. 6, autoradiogram, lane 3) and the binding was inhibited by unlabelled APP (Fig. 6, autoradiogram, lane 4). The incomplete dimerization of protein H in the presence of an excess of APP (Fig. 6, stain, lane 4) is probably due to binding of the peptide to protein H monomers, whereby dimerization and subsequent covalent cross-linking is disturbed. Whereas the experiments with APP demonstrate a physical interaction with protein H, unrelated peptide was not cross-linked with protein H (Fig. 6 autoradiogram, lane 5). This peptide corresponds to one of the Ig light chain-binding domains of protein L, another bacterial surface protein. These results show that the APP sequence is part of the self-associating region of protein H and that the APP peptide can interact with this region.

**Example 4** *The APP sequence is related to bacterial aggregation, adherence and resistance to phagocytosis*

To investigate whether the APP sequence also influences bacterial aggregation, the APP peptide was added to AP1 cultures. APP, and proteins H and M1, reduced the sedimentation rate (Table below), whereas a peptide from the COOH-terminal part of protein H and protein L-derived peptides were without effect.



-23-

**Table 1*****Inhibition of the aggregation of AP1 (% of OD drop after 1h)<sup>1</sup>***

	<u>Protein/peptide</u>	<u>nmol/ml</u>	<u>% Inhibition</u>
	Protein H	1.7	99.5
5	M1 protein	1.3	98.6
	APP	82.5	51.5
	Protein H peptide 351-376	64.6	0
	Protein L, B1-B4	5.0	0
	Protein L peptide 1	150.8	0
10	Protein L peptide 2	82.4	0

<sup>1</sup> AP1 was grown in TH over night in the presence of various proteins/peptides.

The sedimentation was measured and the decrease in optical density (OD drop) after 1 h was calculated and compared to the sedimentation of AP1 in TH alone. Mean values from at least two experiments are given.

APP-related sequences were identified in several M and M-like proteins, including the M1 protein (Fig. 7). Among M serotypes in which APP-related sequences were identified, three additional strains of M serotypes 4, 12 and 49 were tested and found to aggregate, an aggregation that could be blocked with protein H (data not shown). Although many M and M-like proteins remain to be sequenced, the proteins containing the APP-related sequences listed in Figure 7, are found in some of the most frequent and important Streptococcal strains and in particular *S. Pyogenes* serotypes. The related serotypes set out in Fig. 7 represent 46 percent of clinical isolates of *S. pyogenes* in the UK during the period 1980-1990 Colman *et al* J. Med Microbiol 1993 Vol. 39 165-178. Thus, APP-related sequences are very common in clinical isolates implicating that aggregation contributes to the pathogenicity of *S. Pyogenes*, and other Streptococci.

Adherence to mucosal surfaces is an important and early step in *S. Pyogenes* infections. We therefore analyzed the significance of APP-related sequences at the streptococcal surface for aggregation and adherence, using mutants of the AP1 strain and the M6 protein-expressing strain JRS4 which is protein H deficient.

-24-

Detroit 562 human (carcinoma) pharynx epithelial cells (ATCC CCL 138) were cultured in Minimal essential medium with Earle's salt (MEM) from ICN, supplemented with 0.1 mM glutamine (ICN), 10% fetal calf serum (FCS) from Life Technologies and penicillin/streptomycin (5000 units/ml; 5000 µg/ml, PEST; ICN) at 37°C in an atmosphere containing 5% CO<sub>2</sub>. For adhesion assays cells were grown in 24-well tissue culture plates to almost confluence. Cells were washed three times with antibiotic free medium before use. Bacteria were grown in TH at 37°C over night, collected by centrifugation, washed once in PBS and resuspended in MEM supplemented with 10 FCS. 2 x 10<sup>7</sup> bacteria were added per well and plates were incubated for 2h at 37°C. The wells were washed three times with PBS to remove non-adherent bacteria 0.1 ml trypsin (2.5 mg/ml in PBS) and 0.5 ml Triton X-100 (0.025% in PBS) were added to each well for lysis of the epithelial cells. To determine the number of viable bacteria appropriate dilutions were plated, on TH plates, in triplicate from each well.

As shown in Figure 8 both wild-type strains are highly aggregative and they also adhere well to epithelial cells. The BM27.6 is a mutant of AP1 which does not express protein H, whereas BJM71 lacks both protein H and M1 protein. The data of Figure 8 demonstrate that bacterial aggregation and adherence are significantly reduced when protein H is missing at the bacterial surface, and that the removal of also M1 protein further decreases aggregation. The results with the M6 protein-negative mutant JRS145 also suggest that the presence of surface proteins containing APP-related sequences promotes aggregation and adherence.

Like most M protein-expressing strains of *S. pyogenes*, the AP1 strain studied here survives and multiplies in human whole blood. This is in contrast to the non-aggregating BMJ71 mutant which is rapidly killed. As demonstrated above, soluble protein H and APP both interfere with AP1 aggregation. If aggregation contributes to the anti-phagocytic effect, these peptides should inhibit growth of AP1 bacteria in human blood.

Bacteria were grown to early midlog-phase (OD<sub>620</sub>=0.15) and serially diluted in TH. 100 µl of the bacterial solution were incubated with proteins or peptides for 30 min. on ice, mixed with 1 ml heparin-treated blood from different donors, and rotated end over end at 37°C. Samples of 100 µl were drawn at different times, added to 2.5 ml

-25-

TH with 0.5% agar, spread on TH-agar plates, and incubated at 37°C overnight.

As shown in Table 2 below the addition of protein H or APP, but not an unrelated peptide, reduced the multiplication of AP1 bacteria in human whole blood, suggesting that APP-mediated aggregation protects *S. pyogenes* against phagocytosis.

**Table 2** *Survival of AP1 in human blood*

		Time of incubation (h)		
		0	5	
		CFU/ml blood <sup>1</sup>		% Inhibition
	AP1 + PBS	88 ± 67	126476 ± 90500	
10	AP1 + protein H (1.25 nmol)	72 ± 86	52632 ± 60627	57.4±29.4
	AP1 + APP (1000 nmol)	88 ± 82	43938 ± 39205	65.9±25.1
15	AP1 + protein L (1.67 nmol)	173 ± 118	256333 ± 102372	0

<sup>1</sup> Values are mean ± SD from five experiments using blood from three different donors.

#### **Example 6** *Immunisation studies*

Subsequently, studies were carried out to investigate the ability of APP to generate antibodies, indicative of its potential as a vaccine candidate.

A. 19-mer APP-peptide conjugated to KLH (Pierce) was used for raising of antibodies in rabbits. 0.4 mg of 19-mer APP-peptide and 0.1 mg KLH, in a volume of 0.5 ml, were mixed with 0.5 ml Freund's adjuvant (Sigma, St. Louis, MO) (1 part complete and 2 parts incomplete) and 10 x 0.1 ml were injected subcutaneously in the back of a rabbit. Immunisation was done on day 1, and a booster of the same amount of protein was injected in the same way as above at day 30 and day 60.

Each well in a 96-microwell plate (Nunc, Maxisorp) was coated overnight at +4°C with 200 µl of a 1 µg/ml solution of respectively protein, protein H, APP-peptide and protein L-peptide (Actigen, Cambridge UK) diluted in coupling buffer (0.016 M Na<sub>2</sub>CO<sub>3</sub>, 0.035 M NaHCO<sub>3</sub> pH 9.6). After washing, in PBS, the wells were incubated with 200 µl preimmuniserum or antiserum for 1 h at 37°C. The sera were serially

-26-

diluted, 1:1000; 1:2000; 1:4000; 1:8000; 1:16000; 1:32000; 1:64000 and 1:128000, in PBS + 0.05% Tween™-20, 2% bovine serum albumin (BSA).

A secondary antibody, goat anti-rabbit HRP-labelled (BioRad, Hercules, CA) diluted 1:3000 in PBS + 0.05% Tween™-20, 2% BSA was added for detection of activity. 200 mg of ABTS (2, 2'-Azino-di (3-ethylbensthiazolinsulfonat (NH<sub>4</sub>)<sub>2</sub>-salt was dissolved in 10 water. A colour reagent mixture consisting of 40 ml substrate buffer (0.1 M citric acid, 0.1 M Na<sub>2</sub>HPO<sub>4</sub> x 2H<sub>2</sub>O, pH 4.5), 2ml ABTS solution, and 0.8 ml of 30% H<sub>2</sub>O<sub>2</sub> was prepared.

The plate was developed by adding 200 µl of colour reagent mixture to each well followed by incubation for 30 minutes at 37°C. The plates were read in an ELISA reader at 405 nm.

As shown in Figure 9, APP bound antibody confirming generation of anti-APP antibodies.

B. Sheep were primed with 500µg Spy-PH YQE33 (YQWQLKQKQQLETEKQISEASRKSLSRDLEASRC-COOH) conjugated to Keyhole Limpet Haemocyanin (KLH) administered subcutaneously in Freund's complete adjuvant at 6 individual sites. On days 28, 56 and 82 following primary immunisation animals were boosted with 350µg Spy-PH YQE33-KLH conjugate administered subcutaneously in Freund's complete adjuvant at 6 individual sites. Fourteen days after the final booster animals were bled and sera prepared for determination of anti-Spy-PH-YQE33 antibodies by ELISA.

Microtitre plates (96 well) were coated with Spy-PH-YQE33 peptide by addition of 5 µg/ml peptide solution in 0.05 M bicarbonate buffer (pH 9.6) and incubation (60 min, 37°C). Plates were blocked (60 min, 37°C) with 1% bovine serum albumin (BSA) in PBS supplemented with 0.05% Tween-20 (PBS-Tween) to minimise background binding. Sheep immune serum prepared as described above was diluted 1:10000 in PBS-Tween and pre-incubated (60 min, 37°C) with 100 µg/ml Spy-PH-QKQ19 (QKQQQLETEKQLSEASRKSC-COOH) relevant peptide or 100µg/ml Spy-LP-KEY17 (CKEY TDKLDKLDKESKDK-COOH) irrelevant peptide. Control serum was incubated in the absence of peptide. Pre-incubated sera was added to the plates and incubated for 60 min at 37°C. Donkey anti-sheep IgG conjugated to horse radish

-27-

peroxidase (1:1000 dilution in PBS-Tween) was added to the plate (60 min, 37°C) followed by enzyme substrate solution containing 3,3',5,5'-tetramethyl-benzidine in phosphate citrate buffer at pH 5.0 (10 min, RT). The colorimetric reaction was stopped by addition of 2 M HCl and optical density determined at 450 nm. Plates were washed  
5 between each stage of the ELISA for a minimum of 3 times with PBS-Tween.

The results are shown in Figure 12.

C. Recombinant protein H was obtained by expression in *E. coli* as described in Berge et al., (1997) J. Biol. Chem. 272, 20774-20781. Protein H was purified from *E. coli* cell lysates in a single affinity chromatography step using a human  
10 IgG-Sepharose column. Bound protein H was eluted with 3 M KSCN and dialysed against PBS.

Protein extracts were prepared from *S. pyogenes* by partial digestion of the cell wall with lysozyme and mutanolysin. Approximately 2 ml of cells grown to OD<sub>600nm</sub> of 1.0 were pelleted by low speed centrifugation and resuspended in 100 µl of 10 mM  
15 Tris-HCl containing 5mg/ml of freshly added lysozyme (Sigma) and 100 U/ml of mutanolysin (Sigma). After 10 min incubation at 37 °C the cells were recovered by centrifugation and washed twice with 1 ml of 10 mM Tris-HCl (pH 8.0) and then lysed by the addition of 150 µl of NuPage sample buffer (Novex) containing 50 mM DTT.

Proteins were electrophoretically separated using 10% NuPage Bis-Tris-HCl  
20 buffered acrylamide gels and the NuPage MES SDS running buffer to optimise separation proteins in molecular weight range 39 to 60 kDa. Details of the NuPage electrophoresis system can be obtained from Novex Electrophoresis GmbH, Brueningstrasse 50, Building C 584, D-65929 Frankfurt/M. The proteins were transferred to PVDF membrane filters using the Novex XCell II blotting apparatus and  
25 the conditions recommended by the manufacturer. After transfer the PVDF filters were blocked using a 1:1 mixture of UHT virtually fat free milk and phosphate buffered saline containing 0.05% v/v Tween 20 (PBST) for 30 min at room temperature. The filters were incubated with the primary antibody serum (1:1000) in blocking buffer (1 part fat free milk to 3 parts PBST) for 1 h at room temperature and then washed for 3 x  
30 5 min in blocking buffer. The filters were incubated with the anti-sheep IgG alkaline phosphate conjugate diluted 1:5000 in blocking buffer for 30 min at room temperature.

-28-

The filters were finally washed for 2 x 10 min with PBST and 2 x 5 min with 10 mM Tris-HCl (pH 8.0), 150 mM NaCl before addition of bromo-chloro-indolyl phosphate / nitro-blue tetrazolium (BCIP/NBT substrate kit, Sigma) a chromogenic substrate for alkaline phosphatase.

5 Immunoblotting was carried out with recombinant protein H (800 ng) and protein extracts from M6 type (non-protein H expressor) and AP1 (M1 type protein H expressor) strains of *S. pyogenes*. A major band was detected in the tracks loaded with purified recombinant protein H and a slightly higher molecular weight band was detected in the track loaded with AP1 extracts that may represent protein H  
10 enzymatically released in small quantities from the cell wall of strain AP1.

These results demonstrate that immunisation with APP peptide conjugated to carrier protein KLH elicits IgG serum antibodies that bind to purified recombinant protein H and wild type protein H from *S. pyogenes*. Differences in the molecular weight of purified recombinant protein H and wild type protein H from *S. pyogenes* can  
15 be accounted for by the fact that recombinant protein H is 30 amino acids shorter in length.

#### Example 7 Bactericidal Analysis

*Streptococcus pyogenes* group A (AP1) bacteria were grown to early midlog-phase ( $OD_{620} = 0.15$ ) in Todd Hewitt broth (TH) (Difco, Difco Laboratories, Detroit,  
20 MN) at 37°C in 5% CO<sub>2</sub>. The bacteria were serially diluted five times in TH-broth, 0.4 ml bacteria + 4.6 ml TH. 100 µl of the fourth or fifth dilution was incubated, 30 min. on ice, with 100 µl of undiluted preimmunserum (pre-APP) or 100 µl of undiluted antiserum (anti-APP), 100 µl of 1 mg/ml Ig-fractions of preimmunserum (pre-IgG) or antiserum (anti-IgG). 1 ml heparin-treated human blood was added to the 200 µl  
25 mixture and rotated end over end at 37°C.

At various time points samples of 100 µl were drawn, added to 2.5 ml TH with 0.5% agar, spread on TH-agar plates and incubated at 37°C overnight. Colony forming units were counted. IgG-fractions from preimmunserum and antiserum respectively were purified on protein LG-Sepharose.

30 The results are shown in Table 3 below, demonstrating the ability of anti-APP serum to inhibit the survival of AP1 in human blood. APP itself also has an inhibiting activity, outlining the potential of APP not only as a vaccine candidate but also as an

-29-

anti-aggregative agent.

**Table 3****Inhibition of the survival of AP1 in human blood**

5	<b><u>Inhibitor</u></b>	<b><u>% Inhibition</u></b>
	pre-APP serum <sup>1</sup>	30.9 ± 24.8
	anti-APP serum <sup>1</sup>	35.5 ± 15.9
	IgG <sup>2</sup> affinity purified from pre-APP serum	50.3 ± 28.9
	IgG <sup>2</sup> affinity purified from anti-APP serum	80.2 ± 11.8

10 <sup>1</sup> Mean values from five experiments using blood from three different donors

<sup>2</sup> Mean values from three experiments using blood from two different donors

**Example 8 Challenge study with APP-peptide and *S. pyogenes* bacteria**

The usefulness of APP in increasing survival through its anti-aggregative properties was investigated.

15 A *S. pyogenes* strain AP1 of M1 serotype were grown in Todd Hewitt culture medium overnight at 37°C in 5% CO<sub>2</sub> atmosphere. Bacteria were harvested by centrifugation at 3,800 x g for 10 minutes. The resulting bacterial pellet was washed twice and resuspended in 1 x phosphate buffered saline, PBS. A total of 2 mg 19-mer APP-peptide, dissolved in PBS, and 10<sup>6</sup> colony forming units of AP1 bacteria were

20 mixed in a volume of 0.2 ml. This mixture was then injected subcutaneously into mice, using the so-called air sac model. In this method, a syringe is filled and mixed with 0.8 ml air and the 0.2 ml bacteria/peptide mixture. This creates the so-called air-sac model. The mice were left in their cages and survival was monitored over time. The Results are set out in Table 4 below.

**Table 4**

Bacteria (cfu/ml)	Bacteria + APP-peptide	Survival
10 <sup>6</sup>	-	all died
10 <sup>6</sup>	2 mg	all survived

25 The mice that received only bacteria died within 24 hours, while mice that were

30 given bacteria plus APP-peptide were still alive 4 weeks later.

-30-

## CLAIMS

1. A polypeptide of up to 50 amino acids in length, suitable for use as a vaccine against a streptococcal infection, comprising:
  - 5 (a) the amino acids 150-168 of protein H of *S. pyogenes* having the sequence QKQQQLETEKQISEASRKS;
  - (b) an amino acid sequence of an outer membrane protein of a streptococcal strain corresponding to sequence (a);
  - (c) a fragment of sequence (a) or (b) of 6 or more amino acids; or
  - 10 (d) a sequence comprising a sequence (a), (b) or (c) modified by deletion, insertion, substitution or rearrangement.
2. A polypeptide according to Claim 1 wherein the sequence (a) comprises the sequence YQEQLQKQQQLETEKQISEASRKSLSRDLEASR.
- 15 3. A polypeptide according to Claim 1 or 2 wherein the sequence (b) comprises an amino acid sequence of an outer membrane protein of *S. pyogenes*.
4. A polypeptide according to claim 3 wherein the sequence (b) comprises an amino acid sequence of an M or M-like protein of *S. pyogenes*.
- 20 5. A polypeptide according to any one of Claims 1 to 4 wherein the polypeptide is up to 40 amino acids in length and preferably up to 30 amino acids in length.
- 25 6. A polypeptide according to any preceding claim wherein the fragment of sequence (a) or (b) is 10 or more amino acids in length and preferably 18 amino acids in length.
- 30 7. A polypeptide according to Claim 1 wherein the sequence (a) consists essentially of the sequence QKQQQLETEKQISEASRKS or YQEQLQKQQQLETEKQISEASRKSLSRDLEASR.



-31-

8. A chimeric protein comprising a first polypeptide according to any one of the preceding claims and a second polypeptide which is not naturally contiguous to the first polypeptide.

5 9. A polynucleotide encoding the peptide of any one of the preceding claims.

10 10. An expression vector comprising the polynucleotide according to Claim 9 and regulatory sequences operably linked to said polynucleotide for expression of a polypeptide encoded by said polynucleotide.

11. A host cell transfected with the expression vector of Claim 10.

15 12. A pharmaceutical composition comprising a polypeptide according to any of Claims 1 to 8 together with a pharmaceutically acceptable carrier.

13. A vaccine composition comprising a polypeptide according to Claims 1 to 8, an adjuvant and a pharmaceutically acceptable carrier.

20 14. A vaccine composition comprising the polynucleotide of Claim 9 and a pharmaceutically acceptable carrier for said polynucleotide.

15. An antibody against a polypeptide according to any one of Claims 1 to 7.

25 16. A polypeptide of up to 50 amino acids in length comprising:  
(a) the amino acids 150-168 of protein H of *S. pyogenes* having the sequence QKQQQLETEKQISEASRKS;

30 (b) an amino acid of an outer membrane protein of a streptococcus strain corresponding to sequence (a), said sequence having the ability to interfere with aggregation or adhesion of said streptococcal strain;

(c) a fragment of sequence (a) or (b) of 6 or more amino acids, which retains the ability to interfere with streptococcal aggregation or adhesion; and

-32-

(d) a sequence comprising sequences (a), (b) or (c) modified by deletion, insertion, substitution or rearrangement, the sequence retaining the ability to interfere with streptococcal aggregation or adherence.

1/10

Fig.1A.

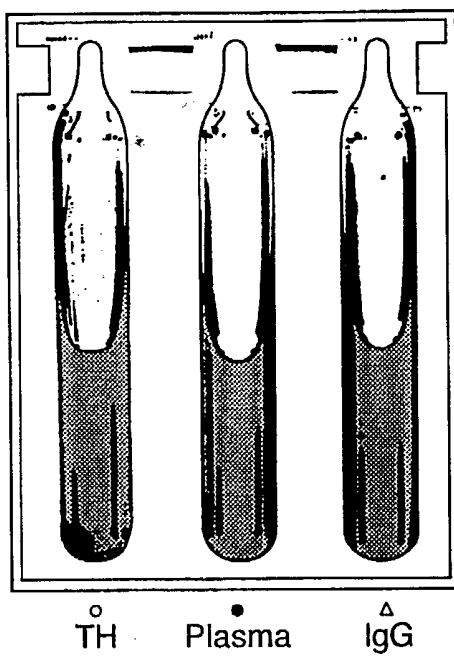
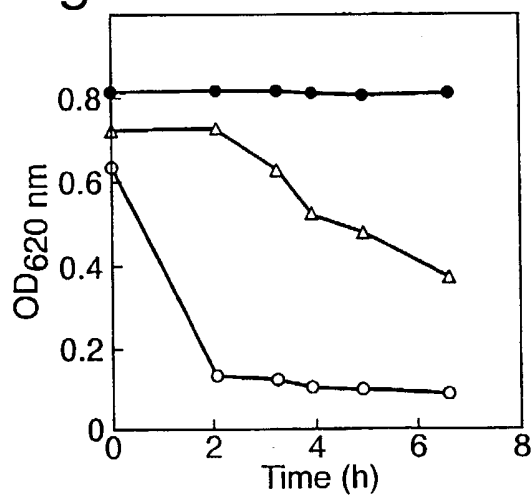
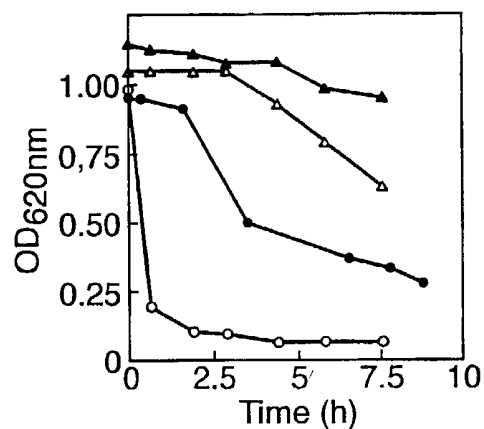
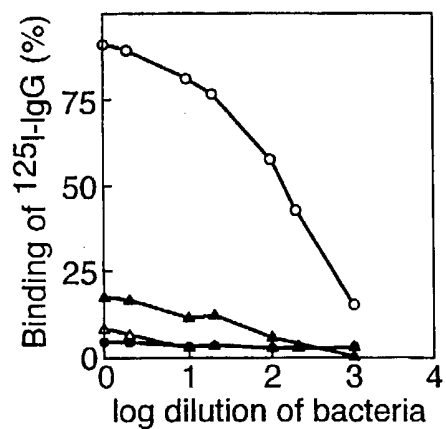


Fig.1B.

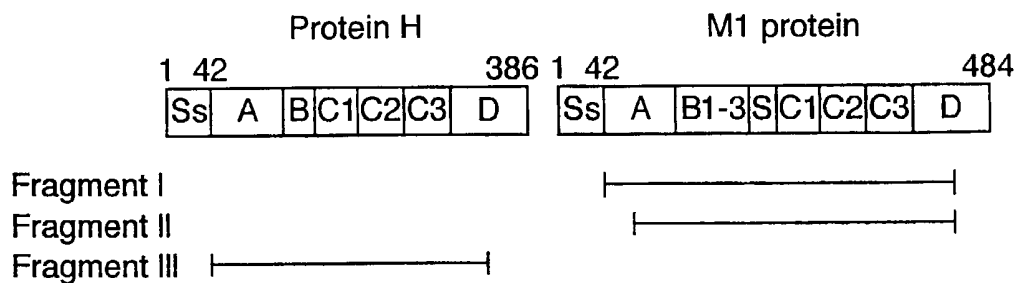
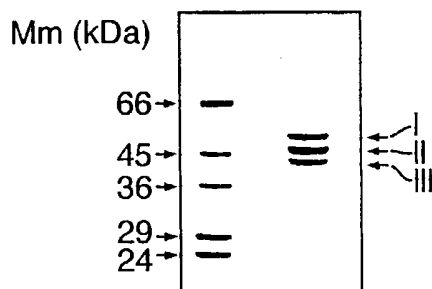




**Fig.2B.**



**Fig.2C.**



3/10

Fig.3A.

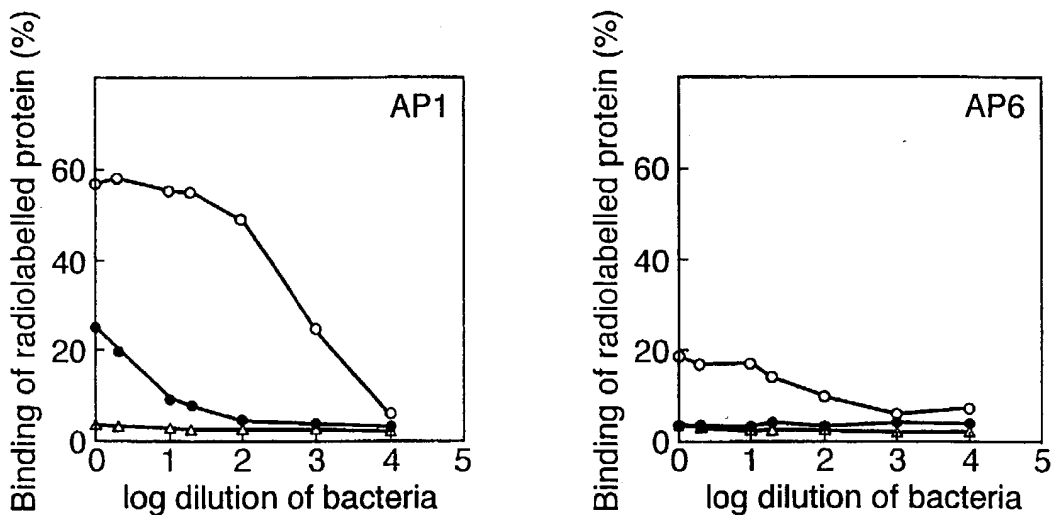
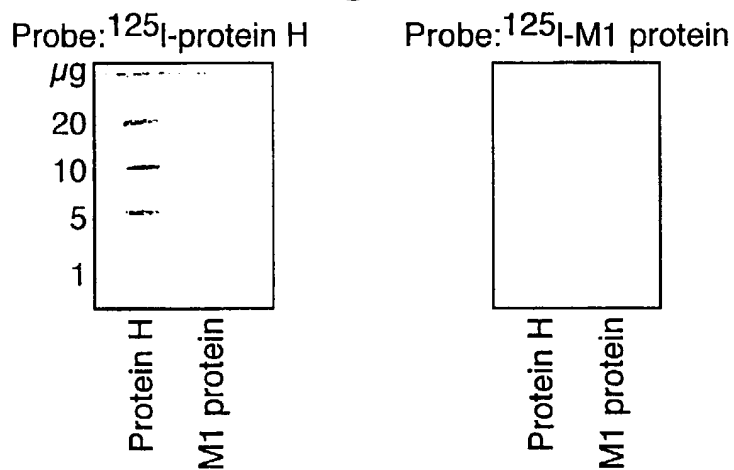


Fig.3B.



4/10

Fig.4A.

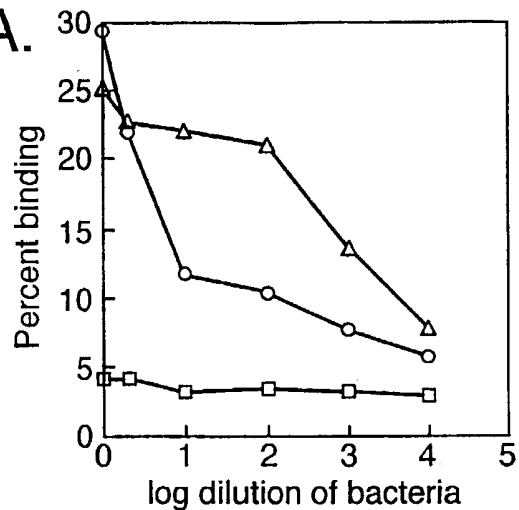


Fig.4B.

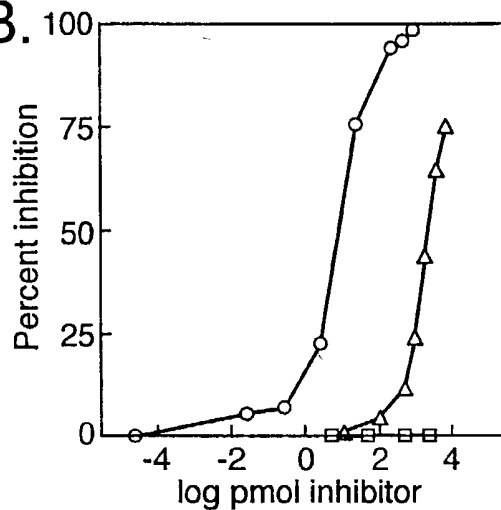
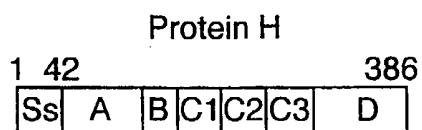


Fig.4C.



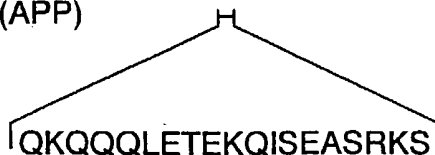
Fragment AB/42-168



Fragment A/42-118



Peptide 150-168 (APP)



5/10

Fig.5.

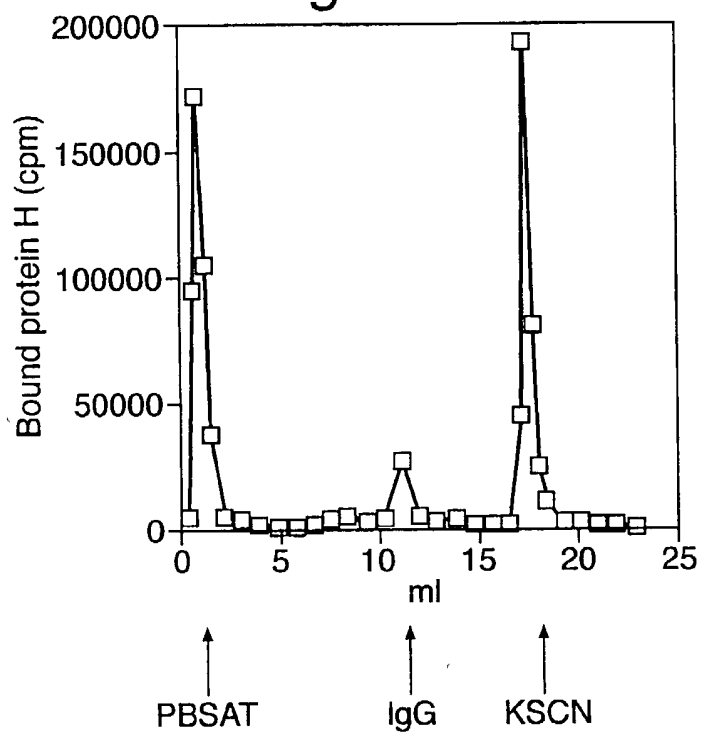
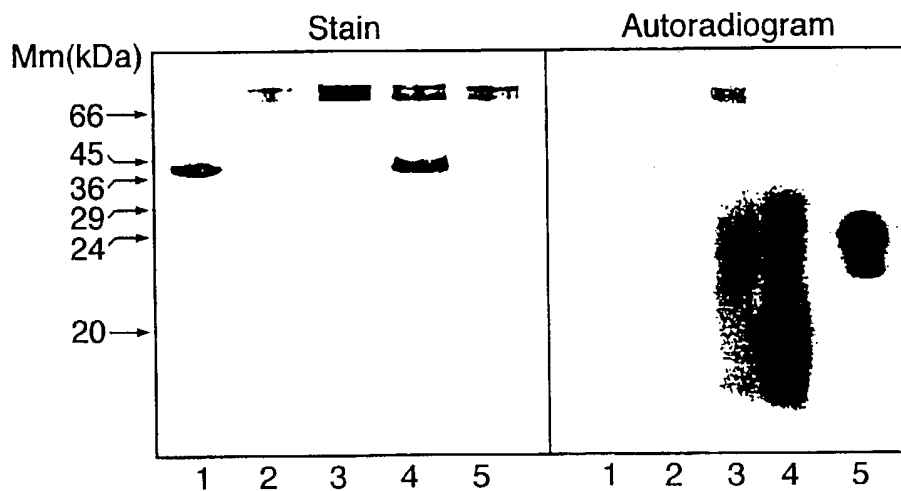


Fig.6.



## Fig. 7.

Peptide sequences homologous to the aggregative protein H peptide 150-168 (APP)

<u>Protein</u>	<u>M serotype</u>	<u>Sequence</u>	<u>% Identity at amino acid level</u>	<u>% Identity at nucleotide level</u>
Protein H/150-168	M1	QKQQQ	100	100
M49/151-169	M49	QKQQQ	100	100
Protein Sir 22/169-187	M22	QKQQQ	100	100
ML2.1/148-166	M2	QKQQQ	95	97
M1/226-244	M1	IEKAK	58	68
M5/318-335	M5	AEQQK	58	68
M12/282-300	M12	GQIKQ	53	63
Protein Arp4/190-208	M4	AEHQK	53	63
M6/309-327	M6	AELDK	42	57
		LETEKQISEA		
		SRKS		
		LETEKQISEA		
		SRKS		
		LETEKQISEA		
		SRKS		
		LEKEKQISEA		
		SRKS		
		LEEEKQISDA		
		SRQS		
		LEEQNKISEA		
		SRKG		
		LEEQNKISEA		
		SRKG		
		LKEEKQISDA		
		SRQG		
		VKEEKQISDA		
		SRQG		

6/10



7/10

Fig.8A.

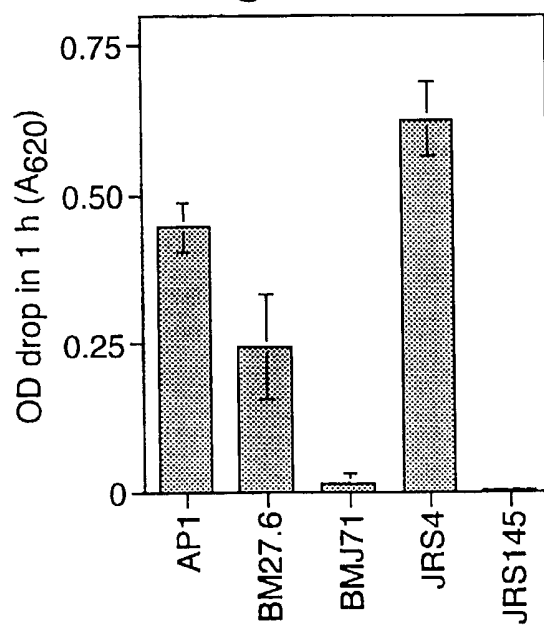
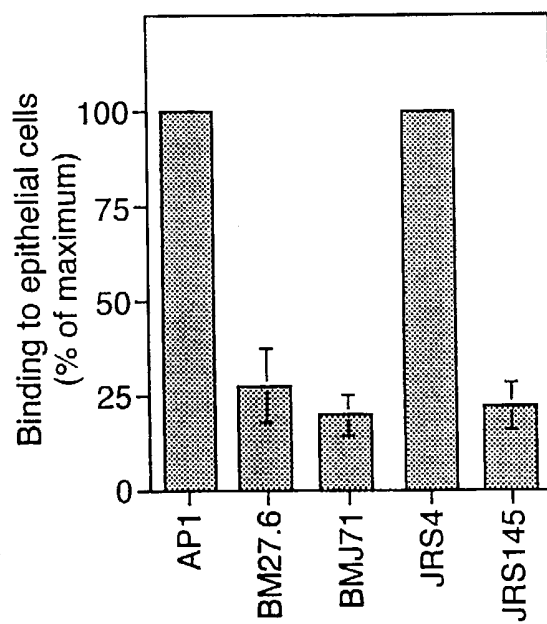


Fig.8B.



8/10

Fig.9.

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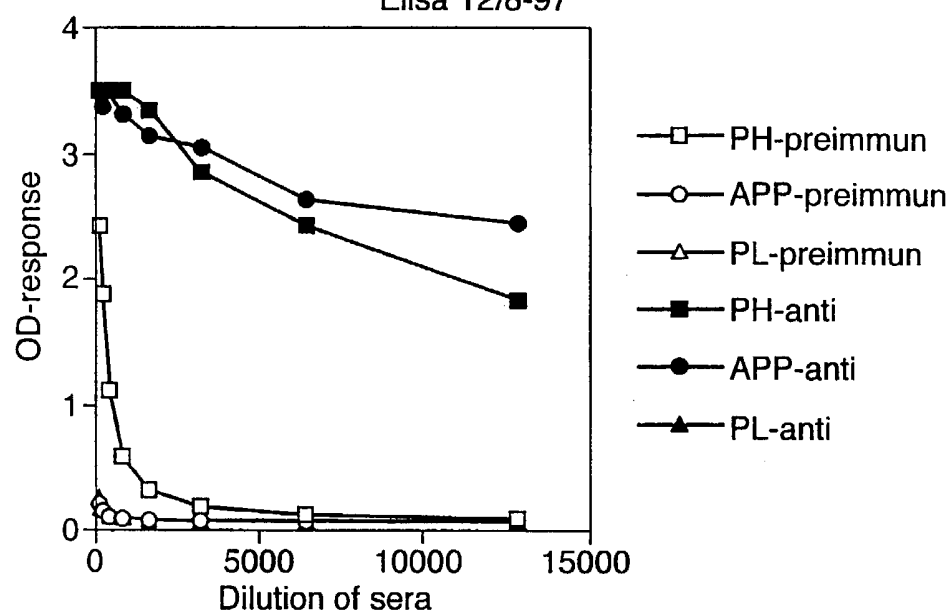
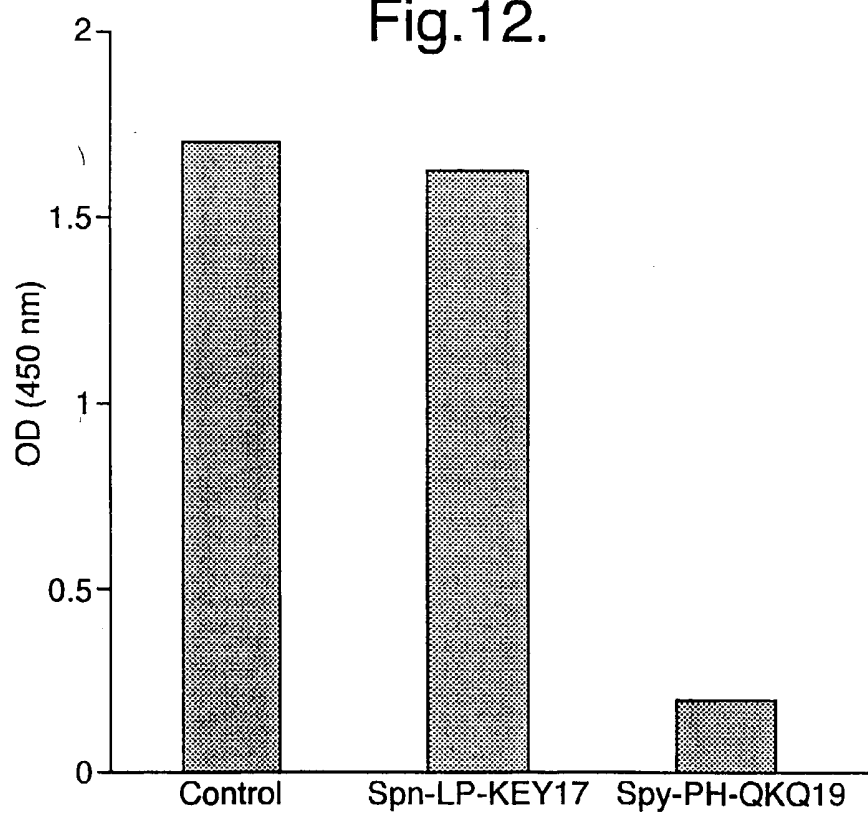


Fig.12.



**Fig.10.** Nucleotide sequence of APP-peptide (33-mer) and homology to other M proteins

1					50
pHapp.seq	TATCAAGAAC	AAC TCCAAA	ACAACAACAA	TTAGAAACAG	AAAAGCAAAT
arp4.seq	AAAAAAGAGC	TTGAAGCTGA	GCACCAAAAA	CTCAAAAGAGG	AAAACAAAT
emm50.seq	TATCAAGAAC	AAC TCCAAA	ACAACAACAA	TTAGAAACAG	AAAAGCAAAT
m1.seq	AAAGAGCAGC	TAACGATCGA	AAAAGCAAAA	CTTGAGGAAG	AAAACAAAT
m12.seq	CTAGCAGAAA	AAGATGGACA	AATCAACAA	CTAGAAAGAGC	AAAACAAAT
m15.seq	TATCAAGAAC	AAC TCCAAA	ACAACAACAA	TTAGAAACAG	AAAAGCAAAT
m24.seq	AAGAAACAAT	TAGAAGCTGA	ACACCAAAAA	CTAGAAAGAAC	AAAACAAGAT
m49.seq	TATCAAGAAC	AAC TCCAAA	ACAACAACAA	TTAGAAACAG	AAAAGCAAAT
m5.seq	AAGAAGCAAT	TAGAAGCTGA	ACAACA AAAA	CTTGAAGAAC	AAAACAAGAT
m6.seq	TTAGCAAACT	TGACTGCTGA	ACTTGATAAG	GTTAAAGGAAG	AAAACAAAT
m9.seq	TATAAAGAAC	AAC TCCACAA	ACAACAACAA	TTAGAAACAG	AAAAGCAAAT
m121.seq	TATCAAGAAC	AAC TCCAAA	ACAACAACAA	TTAGAAACAG	AAAAGCAAAT
sir22.seq	TATCAAGAAC	AAC TCCAAA	ACAACAACAA	TTAGAAACAG	AAAAGCAAAT
	51				99
pHapp.seq	CTCAGAAGCT	AGTCGTAAGA	GCCTAAGCCG	TGACCTTGAA	GCGTCTCGT
arp4.seq	CTCAGACGCA	AGCCGTCAAG	GTCTAAGCCG	TGACCTTGAA	GCGTCTCGC
emm50.seq	CTCAGAAGCT	AGTCGTAAGA	GCCTAAGCCG	TGACCTTGAA	GCGTCTCGT
m1.seq	CTCAGACGCA	AGTCGTCAA	GCCTTCGTG	TGACTTGGAC	GCATCACGT
m12.seq	CCTAGATGCT	AGCCGTAAAG	GTACAGCACG	AGACCTTGAA	GCTGTTCCG
m15.seq	CTCAGAAGCT	AGTCGTAAGA	GCCTAAGCCG	TGACCTTGAA	GCGTCTCGT
m24.seq	TTCAGAAGCA	AGCCGTCAA	GTCTTCGTG	TGACTTGGAC	GCATCACGT
m49.seq	CTCAGAAGCT	AGTCGTAAGA	GCCTAAGCCG	TGACCTTGAA	GCGTCTCGC
m5.seq	TTCAGAAGCA	AGTCGCAAA	GCCTTCGCCG	TGATTTAGAC	GCATCACGT
m6.seq	CTCAGACGCA	AGCCGTCAAG	GTCTTCGCCG	TGACTTGGAC	GCATCACGT
m9.seq	CTCAGAAGCT	AGTCGTAAGA	GCCTAAGCCG	TGACCTTGAA	GCGTCTCGC
m121.seq	CTCAGAAGCT	AGCCGTAAAG	GCCTAAGCCG	TGACCTTGAA	GCTTCTCGT
sir22.seq	CTCAGAAGCT	AGTCGTAAGA	GCCTAAGTCG	TGACCTTGAA	GCGTCTCGT

Peptide sequences homologous to the aggregative protein H peptide 145-177 (APP)

<u>Protein</u>	<u>M serotype</u>	<u>Sequence</u>		<u>% Identity</u>
Protein H/145-177	M1	YQEQLQKQQQ	LETEKQISEA	100
M49/146-178	M49	YQEQLQKQQQ	SRKSLSRDLE	100
EmmL15/142-174	M15	YQEQLQKQQQ	ASR	100
Protein Sir 22/164-196	M22	YQEQLQKQQQ	SRKSLSRDLE	100
Emm50/135-167	M50	YQEQLQKQQQ	ASR	97
ML2.1/143-175	M2	YQEQLQKQQQ	SRKSLSRDLE	94
EmmL9/141-173	M9	YKEQLHKQQQ	SRKSLRRDLE	94
Protein Arp4/185-217	M4	KKELEAEHQK	SRQGLSRDLE	61
M1/221-253	M1	KEQLTIEKAK	ASR	55
M5/313-345	M5	KKQLEAEQQK	SRQSLRRDLD	55
M12/277-309	M12	LAEKDGGQIKQ	SRKGLRRDLD	52
M24/225-257	M24	KKQLEAEHQK	SRKGTARDLE	52
M6	M6	LANLTAELDK	SRQSLRRDLD	ASR

# INTERNATIONAL SEARCH REPORT

International Application No

PC1/GB 99/01104

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/315 C12N15/00 A61K39/09 A61K38/16 C07K16/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 91 19740 A (HIGHTECH RECEPTOR AB) 26 December 1991 (1991-12-26) the whole document --- -/--	1-16



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

24 September 1999

Date of mailing of the international search report

06/10/1999

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Panzica, G

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/01104

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